TITLE OF THE INVENTION: β-TUBULIN INHIBITORS

Field of the invention

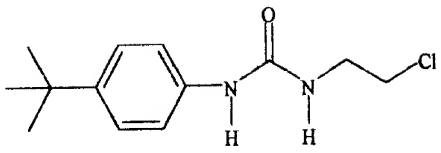
The present invention relates to anticancer molecules that inhibit β-tubulin and that illustrate lower systemic toxicity, higher therapeutic index and a lower capacity to induce resistant phenotypes, which would greatly improve chemotherapy. More specifically, the present invention is directed to the use of various derivatives of 1-aryl-3-(2-chloroethyl)ureas as β-tubulin inhibitors. The present invention is also concerned with a method of selectively attacking cancer cell key proteins by providing derivatives of 1-aryl-3-(2-chloroethyl)ureas having specific spatial configurations allowing these derivatives to dock inside cells at pre-selected sites.

The prior art

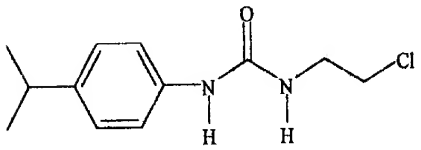
Cancer is a disease state characterized by the uncontrolled proliferation of genetically altered tissue cells. The deleterious effects of most anticancer agents, in combination with the occurrence tumor drug resistance, contribute to failure and relapse of the disease following initial responses to chemotherapy. There have been several chemotherapeutic approaches developed to target cancer including alkylating and anti-mitotic agents, anti-metabolites and anti-tumor antibiotics. Such therapeutic agents act preferentially on rapidly proliferating cells such as cancer cells.

Hormonal therapy using anti-estrogens or anti-androgens constitutes another method of attacking cancer cells.

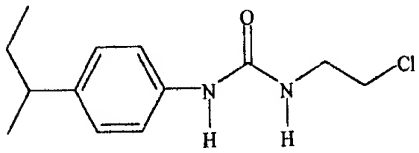
Some 1-aryl-3-(2-chloroethyl)urea derivatives (herein after referred to as "CEUs") are known from US Patents 5,530,026 and 5,750,547 to the same assignee as the present application. More specifically 4-*t*BCEU, 4-*i*PCEU and 4-*s*BCEU are known, whose structures are as illustrated below:



4-*t*BCEU



4-*i*PCEU



4-*s*BCEU

CEUs are also known from PCT application WO 0061546, also to the same assignee as the present application.

It is known that CEUs display affinity towards cancer cells, permeate the cell wall and provide a mild alkylating effect on cell components thereby killing the offending cell.

An object of the present invention is to provide CEU derivatives capable of inhibiting β-tubulin.

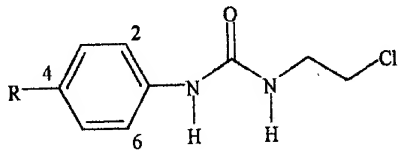
A further object of the present invention is to provide weak monoalkylating agents that are unreactive towards most cellular nucleophiles such as DNA, glutathion and

glutathion reductase but capable of alkylating specific proteins bearing strong nucleophilic centers.

Yet another object of the present invention is to provide prodrugs of the β-tubulin inhibitors as well as inorganic salts thereof. As an example of prodrugs of the compounds of the present invention, the sulfone and sulfoxide derivatives are immediately contemplated by a skilled worker in the art. The sulfone and sulfoxide derivatives while not generally active will be activated once administered to the patient. The activation will occur when the prodrug is reduced to yield the corresponding alkylthio derivative, an active compound.

Summary of the invention.

The present invention provides for β-tubulin inhibitors of formula I, prodrugs thereof and therapeutically acceptable salts thereof,



formula I

wherein R is selected from the group consisting of: *t*-butyl, *i*-propyl and *sec*-butyl. The present invention also provides method of curtailing cancer cell proliferation using a medicament comprising a therapeutically effective amount of the compound of formula 1, prodrugs thereof or therapeutically acceptable salts thereof.

Further scope and applicability of the present invention will become apparent from the detailed description given hereinafter. It should be understood, however, that this detailed description is given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art.

Brief description of the Figures

Fig. 1. Effect of CEU derivatives on cell cycle. Exponentially growing MDA-MB-231 cells were incubated in the absence or presence of 30 μM of either 4-*t*BCEU, 4-*i*PCEU or 4-*s*BCEU for 24 and 48 h, respectively, at 37°C. The cell cycle was evaluated using propidium iodide staining and flow cytometric analysis. Data are representative of three independent experiments.

Fig. 2. Effects of CEU derivatives on microtubule depolymerization. Exponentially growing MDA-MB-231 cells were incubated in the absence or the presence of 30 μM of CEU derivatives including (A) 4-*t*BCEU, 4-*i*PCEU or 4-*s*BCEU for 24 and 48 h respectively; (B) 2-ECEU, 3-ECEU or 4-ECEU for 24 and 48 h; and (C) 4-*t*BEU, 4-*i*PEU, 4-*s*BEU, 4-*t*BCPU, CEU or 4-methoxyCEU for 48 h. Free tubulin and microtubule fractions were isolated and analyzed by Western blot. Data are representative of three different experiments.

Fig. 3. Protein alkylation induced by treatment with [urea-^{14}C]-4-*t*BCEU. (A) Post-nuclei protein extracts from MDA-MB-231 cells incubated with 30 or 100 μM of [urea-^{14}C]-4-*t*BCEU for 12 and 24 h. Panel (B) shows electrophoretic separation of total (T), cytosolic (C) and insoluble (M) proteins, extracted from MDA-MB-231 cells treated with 30 μM [urea-^{14}C]-4-*t*BCEU for 24 h. The protein extracts were analyzed by SDS-PAGE. The gels were transferred onto nitrocellulose membranes and [urea-^{14}C]-4-tBCEU-labeled proteins were revealed by autoradiography. Data are representative of four different experiments. The arrow points to β-tubulin.

Fig. 4. Characterization of the [urea-^{14}C]-4-*t*BCEU-labeled 50-kDa protein by 2-D gel electrophoresis. MDA-MB-231 cells were treated with 30 μM [urea-^{14}C]-4-*t*BCEU for 48 h at 37°C. Total protein extracts were separated by 2-D electrophoresis. The gels were then transferred onto nitrocellulose membranes. (A) The presence of β-tubulin was revealed by immunoblotting; (B) [urea-^{14}C]-4-

*f*BCEU-labeled proteins were visualized by autoradiography. Data are representative of three different experiments.

Fig. 5. Kinetics of β-tubulin alkylation and microtubule disruption induced by [urea-^{14}C]-4-*f*BCEU. Exponentially growing MDA-MB-231 cells were incubated with 30 μM [urea-^{14}C]-4-*f*BCEU for 0, 1, 4, 8, 12, 18, 24 and 48 h. (A) Free tubulin and microtubule fractions were isolated and analyzed by Western blotting; (B) [urea-^{14}C]-4-*f*BCEU-labeled proteins were visualized by autoradiography. Data are representative of three different experiments.

Fig. 6. Identification of the site of alkylation of β-tubulin by CEU: competition with antimicrotubule agents. MDA-MB-231 cells were incubated (A) for 48 h with 30 μM 4-*f*BCEU, 4-*f*PCEU or 4-*s*BCEU in the absence or presence of 5 μM taxol; (B) for 48 h with 30 μM 4-*f*BCEU or 4-sBCEU in the absence or presence of 5 μM colchicine (Col), 5 μM vinblastine (Vbl) or 100 μM iodoacetamide (Iodo); (C) for 24 h with 5 μM colchicine, 5 μM vinblastine or 100 μM iodoacetamide, respectively, and followed by treatment with 100 μM EBI for 2 h. Total protein extracts were analyzed by SDS-PAGE and β-tubulin was revealed by immunoblotting. Data are representative of three different experiments.

Fig. 7. Localization of the site of alkylation by CEU on β-tubulin. Exponentially growing SK-N-SH cells were incubated in the absence or the presence of 30 μM and 100 μM [urea-^{14}C]-4-*f*BCEU for 24 h and 48 h, respectively. Total protein extracts were separated electrophoretically on 10 % polyacrylamide gels. (A) Western blots were performed and βIII-tubulin was revealed with a monoclonal antibody; (B) [urea-^{14}C]-4-*f*BCEU-labeled proteins were visualized by autoradiography. Results are representative of two independent experiments.

Fig. 8. Hypothetical alkylation of β-tubulin by CEU.

Detail d description of preferred mb diments.

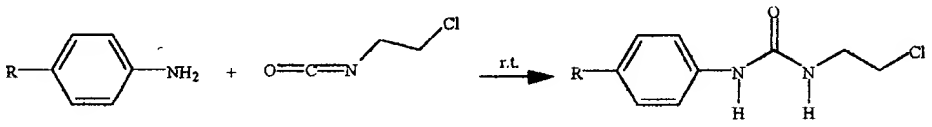
Before describing the present invention in detail, it is to be understood that the invention is not limited in its application to the details of the examples described herein. The invention is capable of other embodiments and of being practiced in various ways. It is also to be understood that the phraseology or terminology used herein is for the purposes of description and not limitation.

Non standard abbreviations used in the paper are: CEU, 1-aryl-3-(2-chloroethyl)urea; 4-*t*BCEU, 4-*tert*-butyl [3-(2-chloroethyl)ureido] benzene; 4-*i*PCEU, 4-*iso*-propyl [3-(2-chloroethyl)ureido] benzene; 4-*s*BCEU, 4-*sec*-butyl [3-(2-chloroethyl)ureido] benzene; 2-ECEU, 2-ethyl [3-(2-chloroethyl)ureido] benzene; 3-ECEU, 3-ethyl [3-(2-chloroethyl)ureido] benzene; 4-ECEU, 4-ethyl [3-(2-chloroethyl)ureido] benzene; 4-*t*BEU, 4-*tert*-butyl [3-(ethyl)ureido] benzene; 4-*i*PEU, 4-iso-propyl [3-(ethyl)ureido] benzene; 4-*s*BEU, 4-*sec*-butyl [3-(ethyl)ureido] benzene; 4-*t*BCPU, 4-*tert*-butyl [3-(2-chloropropyl)ureido] benzene; 4-methoxyCEU, 4-methoxy [3-(2-chloroethyl)ureido] benzene; EBI, N,N'-ethylenebis (iodoacetamide); PBS, phosphate-buffered saline.

Preparation of CEU derivatives

The β-tubulin inhibitors of the present invention are easily prepared in generally good yields. The compounds are also easily purified by usual techniques such as chrystallization or liquid chromatography.

The following reaction sequence illustrates one general scheme of preparation of CEU derivatives of the present invention.



Examples 1 to 3

In accordance to the scheme above, the following 3 example molecules were prepared.

Number	Position, R	R'	Yield (%)	MP (°C)
1	4, *t*-butyl	Chloromethyl	80	133-134
2	4, *i*-propyl	Chloromethyl	65	140-141
3	4, *sec*-butyl	Chloromethyl	95	89-90

^{1}H-NMR analysis

Number	^{1}H NMR (200 MHz) ($CDCl_3$-DMSO-d_6 mixture)
1	7.85 ppm (1, 1H, Ar<u>NH</u>), 7.19 ppm (s, 4H, Ar<u>H</u>$_{2,3,5,}$and $_6$), 6.09 ppm (broad t, 1H (<u>NH</u>CH_2), 3.51 ppm (m, 4H, CH_2<u>CH_2</u>Cl), 1.2 ppm (s, 9H, *t*-butyl)
2	7.87 ppm (1, 1H, Ar<u>NH</u>), 7.16 (dd, 2H, Ar<u>H</u>$_2$ and Ar<u>H</u>$_6$) 6.98 ppm (dd, 2H, Ar<u>H</u>$_3$ and <u>H</u>$_5$), 6.06 ppm (broad t, 1H (<u>NH</u>CH_2), 2.71 ppm (m, 1H, C<u>H</u> $(CH_3)_2$), 1.10 ppm (dd, 6H CH (<u>CH_3</u>)$_2$).
3	7.80 ppm (s, 1H, ArN<u>H</u>), 7.21 ppm (dd, 2H, Ar<u>H</u>$_2$ and Ar<u>H</u>$_6$), 7.00 ppm (dd, 2H, Ar<u>H</u>$_3$ and <u>H</u>$_4$), 6.06 ppm (broad t, 1H (<u>NH</u>CH_2), 3.53 ppm, (m, 4H, CH_2<u>CH_2</u>Cl), 2.46 ppm (sx,1H, C<u>H</u>$(CH_3)CH_2CH_3$), 1.50 ppm (qt, 2H, $CH(CH_3)$C<u>H</u>$_2CH_3$), 1.14 ppm (d, 3H, $CH(CH_3)CH_2CH_3$), 0.74 ppm (t, 3H, $CH(CH_3)CH_2$<u>CH_3</u>).

Evaluation of cytotoxic activity:

The inventors have surprisingly found clear evidence that the CEUs of the present invention are potent antimicrotubule agents that covalently bind to β-tubulin and consequently prevent microtubule assembly.

To better understand the mechanisms responsibl for microtubule disruption by 1-aryl-3-(2-chloroethyl)ureas (CEU), their cytotoxicity was examined on Chinese

hamster ovary cells resistant to vinblastine and colchicine due to the expression of mutated tubulins (CHO-VV 3-2). These cells showed resistance to CEU, e.g. 4-*t*BCEU having an IC_{50} of 21.3 ± 1.1 μM as compared with an IC_{50} of 11.6 ± 0.7 μM for wild-type cells, suggesting a direct effect of the drugs on tubulins. Western blot analysis confirmed the disruption of microtubules and evidenced the formation of an additional immunoreactive β-tubulin with an apparent lower molecular weight on SDS polyacrylamide gel. Incubation of MDA-MB-231 cells with [urea-^{14}C]-4-*t*BCEU revealed the presence of a radioactive protein which coincided with the additional β-tubulin band, indicating that CEU could covalently bind to the β-tubulin. The 4-*t*BCEU-binding site on β-tubulin was identified by competition of the CEU with colchicine, vinblastine and iodoacetamide, a specific alkylating agent of sulfhydryl groups of cysteine residues. Colchicine, but not vinblastine, prevented formation of the additional β-tubulin band, suggesting that 4-*t*BCEU alkylates either Cys239 or Cys354 on β-tubulin.

To determine the cysteine residue alkylated by 4-*t*BCEU, radiolabeled drug was incubated with human neuroblastoma cells (SK-N-SH) which overexpress the βIII-tubulin an isoform where Cys239 is replaced by a serine residue. The results clearly showed that βIII-tubulin is not alkylated by [urea-^{14}C]-4-*t*BCEU, suggesting that cysteine 239 residue is essential for the reactivity of 4-*t*BCEU with β-tubulin. Taken together, these findings indicate that the mechanism of cytotoxicity of CEU involves microtubule depolymerization through alkylation of β-tubulin.

MATERIALS AND METHODS

Cell culture:

Human breast carcinoma cell line, MDA-MB-231, was obtained from the American Type Culture Collection (ATCC HTB-26; Bethesda, MD). MDA-MB-231 cells were grown in RPMI 1640 medium supplemented with 10% bovine calf serum (Hyclone, Road Logan, Utah). Wild-type Chinese Hamster Ovary cells (CHO-10001) (7),

colchicine- and vinblastine- resistant (CHO-VV 3-2) (8) and taxol-resistant (CHO-TAX 5-6) cells (9) were generously provided by Dr. Fernando Cabral (University of Texas Medical School, Houston, Texas). These cells were cultured in RPMI 1640 containing 10% fetal bovine serum. SK-N-SH human neuroblastoma cells, (ATCC HTB-11; Bethesda, MD) were cultured in MEM medium supplemented with 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate and 10% fetal bovine serum. Cells were cultured in a humidified atmosphere at 37°C in 5% CO_2.

Comparative drugs:

Colchicine, vinblastine, taxol and iodoacetamide were purchased from Sigma (St.Louis, MO). CEU derivatives and EBI were prepared as already described (3, 4, 10). Synthesis of [urea-^{14}C]-4-*t*BCEU was carried out as described previously (11). All drugs were dissolved in DMSO and the final concentration of DMSO in the culture medium was maintained at 0.5% (v/v).

Cytotoxiciy assay:

Cytotoxicity was assessed using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) as described by Carmichael et al. (12). Cytotoxic activity of these compounds was expressed as the concentration of CEU inhibiting MDA-MB-231 cell growth by 50% (IC_{50}).

Kinetics of alkylation of 4-(4-nitrobenzyl)pyridine by CEU derivatives:

The rate constant of alkylation (K') of CEU derivatives and chlorambucil was evaluated by a colorimetric assay as described by Bardos et al (13). Briefly, 1 ml of a 10% (v/v) solution of 4-(4-nitrobenzyl)pyridine in ethanol and 1 ml of 50 mM sodium acetate (pH 4.3) were added to an ethanol solution (95%) containing 400 nmol per ml of either chlorambucil or CEU and heated to 80°C in a shaking water bath for 60, 90, 120 or 150 min. The reaction was stopped by cooling the mixtures on ice for 5 min. Then, 1.5 ml of 0.1 M KOH : ethanol (1:2, v/v) were add d to the reaction mixture. Samples were vortexed for 12 sec and set aside for 2.5 min prior to reading the absorbance at 570 nm. The values were compared

with those obtained using a blank sampl containing all reagents except the alkylating agent.

Cell cycle analysis:

Following incubation of MDA-MB-231 cells with 4-*t*BCEU, 4-*i*PCEU or 4-*s*BCEU, the cells were harvested, resuspended in 1 ml PBS and fixed by the addition of 2.4 ml of ice cold anhydrous ethanol. Then, 5 × 10^5 cells from each sample were centrifuged for 3 min at 1000 × *g*. Cell pellets were resuspended in PBS containing 50 µg/ml of PI and 40 U/ml of ribonuclease A (Boehringer Mannheim, Laval, CA). Mixtures were incubated at room temperature for 30 min and cell cycle distribution was analyzed using an Epics Elite ESP flow cytometer (Coulter Corporation, Miami, FL, USA).

Separation of soluble and polymerized tubulins:

Separation of soluble and polymerized tubulins from MDA-MB-231 cells was carried out as described by Minotti et al. (14) with minor modifications. Briefly, after drug exposure, about 5 × 10^6 cells in 100-mm petri dishes were washed with PBS at 37°C and harvested in 3 ml of PBS containing 0.4 µg/ml of taxol using a rubber policeman.

Cells were centrifuged and lysed using 250 µl of microtubule-stabilizing buffer (20 mM Tris-HCl, pH 6.8, 140 mM NaCl, 1 mM $MgCl_2$, 2 mM EDTA, 0.5% Nonidet P-40 and 0.4 µg/ml taxol), and then transferred to 1.5 ml microcentrifuge tubes. Samples were centrifuged at 12,000 × *g* for 10 min at 4°C and the supernatants containing soluble tubulin were placed in separate microcentrifuge tubes containing 250 µl of 2x Laemmli sample buffer (15). Pellets containing the polymerized tubulin were resuspended in 250 µl of water, followed by two freeze/thawing cycles, and the addition of 250 µl of 2× Laemmli sample buffer. Samples were analyzed by SDS-PAGE and immunoassay was performed as described below.

Subc llular fracti nati n of MDA-MB-231 cells.

Cells (~5 × 10^6) were incubated with 30 or 100 μM [^{14}C]-*t*BCEU for 12 or 24 h and then washed with PBS and harv sted by scraping in lysis buffer (5 mM Hepes pH 7.4, 1 mM $MgCl_2$, 10 mM KCl, 1 mM $CaCl_2$, 1 mM PMSF, 1 mM benzamidine and 1 mM aprotinin). Cell lysates were homogenized using a tissue grinder and the final sucrose concentration of each sample was adjusted to 250 mM. Samples were centrifuged at 600 × *g* for 10 min at 4°C to isolate the post-nuclear supernatant. The pellets, containing nuclei and intact cells, were discarded and the post-nuclear supernatant was recentrifuged at 90,000 rpm using a Rotor TLA-100.1 in a Beckman TL-100 ultracentrifuge for 30 min to separate the cytosolic fraction (C) from the insoluble fraction (M) containing the membrane components and mitochondria. One volume of 2× Laemmli sample buffer was then added to the supernatant (C) and the pellet (M) was resuspended in 200 μl of Laemmli sample buffer. Samples were boiled for 5 min and kept at –20°C until analysis.

SDS-PAGE analysis and immunoblotting of β-tubulin:

Samples (1 × 10^5 cells) were analyzed by 10% SDS-PAGE using the Laemmli system (15). Membranes were then incubated with PBSMT (PBS, pH 7.4, 5% fat-free dry milk and 0.1% Tween-20) for 1 h at room temperature, and then with 1:500 monoclonal anti-β-tubulin (clone TUB 2.1, Sigma) or 1:400 anti (clone no. SDL.3D10, Sigma) for 1 h. This monoclonal antibody is specific to βIII-tubulin, and does not cross-react with other β-tubulin isoforms. Membranes were washed with PBSMT and incubated with 1:2500 peroxidase-conjugated anti-mouse immunoglobulin (Amersham Canada, Oakville, Canada) in PBSMT for 30 min. Detection of the immunoblot was carried out with the ECL Western blotting detection reagent kit (Amersham Canada, Oakville, Canada).

Preparation of protein extract and two-dimensional SDS polyacrylamide gel electrophoresis:

MDA-MB-231 cells (~5 × 10^6) incubated with 30 μM [urea-^{14}C]-4-*t*BCEU (11) for 48 h were harvested by scraping and transferred to a 1.5 ml microcentrifug tube. Cell pellets were lysed by addition of 1 ml lysis buffer containing 4% (w/v) CHAPS,

4.6% Ampholines (comprised of 3.6% Ampholine pH range 5 to 7 and 1% Ampholine pH 3 to 10) (Sigma, St-Louis, MO), 50 mM dithiothreitol, 1 mM PMSF and 1 mM benzamidine. Samples were homogenized by 10 passages through a 26G needle and incubated with 1 mg/ml DNase and 0.25 mg/ml RNase A for 5 min on ice. Then, EDTA/EGTA (1:1) and urea were added to final concentrations of 1 mM and 8.5 M, respectively. Samples were centrifuged at 14 000 rpm for 2 min at 4°C and 0.03% (w/v) bromophenol blue was added to the supernatant. Samples were kept at -80°C until processed. Protein extracts were separated by isoelectric focusing according to the procedure described by O'Farrell (16) with minor modifications. Briefly, samples were applied to a 4.5% polyacrylamide gel containing 8.5 M urea, 2% (w/v) CHAPS and 2% (v/v) ampholines. Samples were prefocused at 200, 300 and 400 Volts for 15, 30 and 30 min respectively (17). Isoelectric focusing was performed at 200, 400, 800 and 600 Volts during 0.5, 15, 1 and 1.5 h, respectively. Gels were equilibrated twice for 15 min in buffer A containing 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 2% (v/v) SDS and 2% (w/v) dithiothreitol and for 10 min in buffer B containing 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 2% (v/v) SDS and 0.5% (w/v) iodoacetamide. In the second dimension, proteins were separated according to their molecular weight by using a 10% polyacrylamide SDS gel. The gels were then transferred onto a nitrocellulose membrane.

Detection of proteins alkylated by [urea-^{14}C]-4-*t*BCEU:

Nitrocellulose membranes were dried for 3 days at room temperature and fixed under a FBTIV-816 UV transilluminator at 312 nm (Fisher Scientific, Ottawa, Canada) for 3 min. Membranes were incubated for 1 h in Entensify Aqueous Fluor Solution B (DuPont, Boston, MA), dried and exposed to X-ray film (Kodak, Biomax MR Film) for a week.

RESULTS

Structure-activity relationships between the alkylation potency and the cytotoxicity of CEU:

To evaluate the m chanisms responsible for the cytotoxicity of CEU, first was compared the cytotoxicity and the NBP alkylation constant of various CEU with different IC_{50} ranging from 2 to >140 μM. **Table 1** shows that CEU are weak alkylators of 4-(4-nitrobenzyl)pyridine as compared to chlorambucil (prior art molecule used for comparative purposes).

Table 1: Molecular structure, cytotoxic activity and relative alkylation of 4-(4-nitrobenzyl)pyridine by CEU derivatives.

Concentration inhibiting 50% of cell growth (IC_{50}) as determined graphically from the survival curves. The constant of alkylation (K') was determined by linear regression of the respective curves generated for each drug. Data represent mean values ± SD from at least three independent experiments.

Name	Position, R	R'	IC_{50} (μM) MDA-MB-231	K'[b]
4-*t*BCEU	4, *t*Bu	$-CH_2Cl$	4.6 ± 0.3[a]	3.1 ± 0.3[a]
4-*i*PCEU	4, *i*Pr	$-CH_2Cl$	2.2 ± 0.3[a]	3.0 ± 0.1[a]
4-*s*BCEU	4, *s*Bu	$-CH_2Cl$	2.3 ± 0.1[a]	3.1 ± 0.3[a]
CBL (comparative example)	Chlorambucil		9.3 ± 3.5[a]	38.9 ± 5.1[a]

[a] Values are mean ± SD.
[b] $K' = (A_{t2}-A_{t1}/(t_2-t_1)$, where A= absorption at 570 nm and (t_2-t_1)= period of incubation.
[c] ND, not determined.

As seen from **Table 1** above, the K' of CEU are almost thirteen times lower than those for chlorambucil, a known alkylating agent derived from aromatic nitrogen mustards (18). In addition, the cytotoxicity of different CEU did not correlate with their alkylation potency. Indeed, active CEU such as 4-*t*BCEU, 4-*i*PCEU or 4-*s*BCEU having IC_{50} of 4, 2 and 2 μM respectively, and inactive CEU such as CEU, 2-ECEU, 4-*s*BEU and 4-*t*BCPU, have almost the same K' values (≈2.5 to 3.5 μM). Furthermore, CEU did not show detectable alkylation of either DNA, glutathione or glutathione reductase. However, substitution of the 2-chloroethyl moiety of active CEU analogs with methyl, ethyl or 3-chloropropyl groups

diminish d their cytotoxicity. This suggests that albeit v ry weak, the alkylation potency of CEU is nevertheless involved in the mechanisms of their cytotoxicity.

Differential cytotoxicity induced by 4-*t*BCEU in CHO cell lines expressing mutated tubulin:

The cytotoxicity of 4-*t*BCEU, 4-*i*PCEU and 4-*s*BCEU in two CHO cell lines having differential sensitivity to antimicrotubule agents was evaluated. These cell lines are derived from parental CHO-10001 cells and express mutated tubulins. The CHO-VV 3-2 cell line is resistant to vinblastine and colchicine, and hypersensitive to taxol, while the CHO-TAX 5-6 cell line is resistant to taxol and hypersensitive to vinblastine and colchicine (7-9).

Results are reported in **Table 2** below.

Table 2: Cross-resistance of 4-*t*BCEU in CHO cells expressing mutated tubulin.

Wild-type (10001), vinblastine and colchicine-resistant (VV 3-2), and taxol-resistant (TAX 5-6) CHO cells were incubated in the presence of increasing concentrations of chlorambucil, colchicine, taxol, 4-*t*BCEU, 4-*i*PCEU and 4-*s*BCEU for 120 h. Cell survival was evaluated using the MTT colorimetric assay, 100% survival representing cell viability in the absence of drug. Data are representative from three independent experiments.

	IC_{50} (μM)		
Name	CHO-10001	CHO-TAX 5-6	CHO-VV 3-2
Colchicine	0.0020 ± 0.0003[a]	0.00060 ± 0.00001[a,c]	0.0036 ± 0.0006[a,b]
Vinblastine	0.030 ± 0.003[a]	0.021 ± 0.003[a,c]	0.094 ± 0.003[a,b]
Taxol	1.7 ± 0.3[a]	6.1 ± 0.5[a,b]	1.1 ± 0.2[a,d]
4-*t*BCEU	11.6 ± 0.7[a]	4.6 ± 0.3[a,c]	21.3 ± 1.1[a,b]
4-*i*PCEU	4.4 ± 0.2[a]	2.0 ± 0.1[a,c]	7.7 ± 0.4[a,b]
4-*s*BCEU	5.0 ± 0.2[a]	2.1 ± 0.1[a,c]	8.2 ± 0.4[a,b]

[a] Values are mean ± SD of three determinations.
[b] Significantly more resistant than parental CHO-10001 cells; $P \leq 0.02$, Student t-test.
[c] Significantly more sensitive than parental CHO-10001 cells; $P \leq 0.02$, Student t test.

[d] Not significantly different from parental CHO-10001 cells.

As shown from **Table 2**, the cytotoxicity of 4-*t*BCEU, 4-*i*PCEU and 4-*s*BCEU was higher in CHO-TAX 5-6 cells (eg. IC_{50} = 4.6 ± 0.3 μM for 4-*t*BCEU) and lower in CHO-VV 3-2 cells (eg. IC_{50} = 21.3 ± 1.1 μM for 4-*t*BCEU). The cytotoxicity of 4-*t*BCEU in CHO-10001 cells was 11.6 ± 0.7 μM. These results strongly suggest that the cytotoxicity of CEU is due to microtubule depolymerization.

Effects of CEU on MDA-MB-231 cell cycle:

The effect of CEU, namely 4-*t*BCEU, 4-*i*PCEU and 4-*s*BCEU, on the cell cycle was analyzed. To this end, exponentially growing MDA-MB-231 cells were treated with 30 μM 4-*t*BCEU, 4-*i*PCEU or 4-*s*BCEU for 24 or 48 h followed by an evaluation of cell cycle distribution by flow cytometry using propidium iodide, a fluorescent DNA dye. This flow cytometric analysis allows to determine the proportion of cells in G_0/G_1, S and G_2 + M fractions of the cell cycle but does not allow to distinguish between G_2 and M arrest. As illustrated in **Fig. 1**, 24 h incubation with CEU derivatives caused a significant accumulation of cells in the G_2 + M suggesting that CEU derivatives, like colchicine and vinblastine, induce microtubule disruption and consequently prevent mitosis. Induction of mitosis blockage in prophase by CEU derivatives was confirmed by mitotic index analysis as described by Drouin (22).

Moreover, the cell cycle arrest in G_2 + M was induced more rapidly with 4-*i*PCEU and 4-*s*BCEU than with 4-*t*BCEU. As expected, antimicrotubule agents such as colchicine or vinblastine also lead to a G_2 + M block in MDA-MB-231 cell cycle.

Effect of CEU on the depolymerization of microtubules:

To assess the effects of CEU derivatives on microtubules in MDA-MB-231 cells, the relative levels of polymerized and soluble tubulin in cells using SDS-PAGE analysis and a monoclonal β-tubulin antibody (see Materials and Methods) were determined. **Fig. 2A** shows that complete microtubule depolymerization was observed after incubation with 30 μM 4-*t*BCEU, 4-*i*PCEU or 4-*s*BCEU for 24 h and 48 h. Furthermore, a second immunoreactive band with an apparent lower molecular

weight (M_r) than native β-tubulin was detected. Interestingly, this second immunoreactive band, herein called modified β-tubulin, was only obs rved in the free tubulin fraction. Formation of the modified β-tubulin was induced in a dose-dependent manner by 4-*t*BCEU (data not shown). Furthermore, its presence was specific to active CEU, since no such band was observed with colchicine, vinblastine or taxol (data not shown). Finally, the effect of 4-*t*BCEU seemed to be specific to β-tubulin since no electrophoretic evidence for α-tubulin modification could be found (data not shown). To assess structure-activity relationships between the molecular structure of other CEU derivatives, we compared their relative rates of microtubule depolymerization and formation of the modified β-tubulin. To this end, CEU derivatives were grouped into three sub-classes of activity: **i)** highly active CEU (IC_{50} between 2 and 5 μM) **(Fig. 2A), ii)** weakly active CEU (IC_{50} between 10 to 50 μM) **(Fig. 2B)**, and **iii)** essentially inactive CEU (IC_{50} >50 μM) **(Fig. 2C)**. **Fig. 2A** shows that the effects of the highly active 4-*s*BCEU and 4-*i*PCEU on the formation of modified β-tubulin are slighly faster than for 4-*t*BCEU.

Microtubule depolymerization and the appearance of the modified β-tubulin with CEU of low cytotoxicity such as 3-ECEU and 4-ECEU were delayed in time, if compared to the most cytotoxic CEU **(Fig. 2A).** Moreover, no depolymerization and no effect on β-tubulin were observed with inactive 2-ECEU **(Fig. 2B)** and with CEU having a modified 2-chloroethyl moiety such as 4-*t*BEU, 4-*i*PEU, 4-*s*BEU **(Fig. 2C)**, suggesting that alkylation is required for β-tubulin inactivation and microtubule disruption. Finally, 4-*t*BCPU, as well as unsubstituted CEU and CEU bearing polar groups such as 4-methoxy were also inactive **(Fig. 2C).** When combined these results suggest that alteration of β-tubulin, possibly by alkylation and microtubule disassembly, might at least in part, be responsible for the cytotoxic activity of CEU.

Alkylation of cellular proteins by [urea-^{14}C]-4-*t*BCEU in MDA-MB-231 cells:
Since the appearance of the modified β-tubulin was the hallmark of active CEU, the inventors determined that CEUs could specifically alkylate β-tubulin. MDA-MB-231 cells were incubated with [urea-^{14}C]-4-*t*BCEU and cellular proteins were analyzed

by SDS-PAGE. In **Fig. 3A**, a small number of radiolabel d proteins were detected with main radioactive species with Mr at >200, 50, 34 and 29 kDa, respectively. The appearance of these bands was time- and dose-dependent, suggesting that they are specific targets of the radiolabeled 4-*t*BCEU. Subcellular localization of these proteins indicated that alkylated proteins with M_r at >200 kDa and as well as 34-kDa proteins were found in the insoluble fraction (M) containing the membrane proteins, while the 50-kDa and 29-kDa proteins were found in the cytosolic fraction (C). The protein of 50-kDa is more specifically labeled at low concentrations of [urea-^{14}C]-4-*t*BCEU and the molecular weight of this protein band coincided with that of the modified β-tubulin (data not shown), suggesting that 4-*t*BCEU could alkylate β-tubulin.

To validate this discovery, the inventors carried out a two-dimensional gel electrophoresis experiment on the proteins extracted from cells treated with 30 μM [urea-^{14}C]-4-*t*BCEU for 48 h, (**Fig. 4A** and **B**), while native and modified β-tubulin were revealed by immunoblotting (**Fig. 4A**). As depicted in **Fig. 4B**, the ^{14}C-labeled 50-kDa protein and the modified β-tubulin, co-localized on the gel, indicate that β-tubulin modification likely resulted from direct alkylation by 4-*t*BCEU. This was confirmed by isolation via two-dimensional gel electrophoresis of the 50-kDa ^{14}C-labeled protein or modified β-tubulin and mass spectral analysis using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (23) (data not shown). To determine whether alkylated or modified β-tubulin induce microtubule disassembly, MDA-MB-231 cells were incubated with [urea-^{14}C]-4-*t*BCEU for various time intervals, followed by separation of soluble and polymerized tubulins and SDS-PAGE analysis. Native and modified β-tubulin were detected by a monoclonal β-tubulin antibody (**Fig. 5A**) and whereas ^{14}C-labeled modified β-tubulin was detected by autoradiography (**Fig. 5B**). **Fig. 5A** shows that complete microtubule disruption occurs after 8 to 12 h of incubation whereas modified β-tubulin appears only at 12 h. Interestingly, **Fig. 5B** illustrates that ^{14}C-labeled modified β-tubulin appears after only 1 to 4 h of incubation with the drug. These

results confirm that the alkylation of β-tubulin occurs prior to microtubule disruption, suggesting that β-tubulin alkylation by [urea-^{14}C]-4-*t*BCEU induces microtubule disassembly. Interestingly, alkylated β-tubulin was found solely in the soluble protein fraction, suggesting that the alkylation of β-tubulin is possible only when the protein is in its unpolymerized state. Thus, the site of β-tubulin alkylation by CEU might be involved in the microtubule assembly process, through as yet unknown mechanisms.

Identification of the alkylation site of CEU on β-tubulin:

To identify the site of β-tubulin alkylation by CEU, competition experiments between 4-*t*BCEU, 4-*i*PCEU, 4-*s*BCEU and various antimicrotubule agents such as taxol, colchicine and vinblastine were carried out. The latter agents were used because they have well defined binding sites on β-tubulin (24-28) and because they were expected to inhibit alkylation by CEU if they shared a common binding site. **Fig. 6A** shows that treatment of MDA-MB-231 cells with 4-*t*BCEU, 4-*i*PCEU or 4-*s*BCEU in the presence of taxol, a microtubule stabilizing agent (29, 30), abrogated the formation of modified β-tubulin. Furthermore, immunoblotting experiments (**Fig. 6B**) showed that colchicine, but not vinblastine, prevented the formation of modified β-tubulin, induced by 4-*t*BCEU or 4-*s*BCEU. These data suggest that the binding site of CEU on β-tubulin is in the vicinity of the colchicine binding site. This was confirmed by treatment of cells with [urea-^{14}C]-4-*t*BCEU in presence of colchicine or vinblastine, which showed the inhibition of the formation of the 50-kDa radiolabeled band by the former, but not the latter drug (data not shown). Two potential sites of alkylation have been identified in the vicinity of the colchicine binding site on β-tubulin (25). These putative alkylation sites are two nucleophilic mercapto groups of cysteine residues at positions 239 and 354, respectively (25). To assess the possible alkylation of these mercapto residues by CEU, MDA-MB-231 cells were treated with 4-*t*BCEU in the presence of iodoacetamide, a specific thiol alkylating agent (31, 32). **Fig. 6B** shows that iodoacetamid completely prev nt d β-tubulin alkylation by 4-*t*BCEU, indicating that 4-*t*BCEU alkylates cysteine residues. Similar experiments were carried out using EBI, a bifunctional

alkylating agent that sp cifically induces cross-linking between Cys239 and Cys354 of β-tubulin (32).

This modification altered the electrophoretic behaviour of β-tubulin (32) similar to the modification observed with cytotoxic CEU. Interestingly, the β-tubulin alteration induced by EBI is abrogated by pre-treatment of cells with colchicine or iodoacetamide but not with vinblastine (**Fig. 6C**). These results strongly suggest that EBI and CEU share the same alkylation site on β-tubulin, which is either Cys239 or Cys354 or both.

Localization of the cysteine residue(s) alkylated by CEU:

The results presented above suggest that alkylation of cysteine residues of β-tubulin could occur either on Cys239 or Cys354 or on both residues. Alkylation of both residues is most unlikely since CEU are monoalkylating agents. To discriminate between alkylation of Cys239 or Cys354, we compared the relative alkylation induced by [urea-^{14}C]-4-*f*BCEU on βIII-tubulin isoform (**Fig. 7A**) using an antibody which specifically recognizes βIII-tubulin without cross-reactivity with other tubulin isotypes. βIII-tubulin, is characterized by the substitution of the Cys239 residue by a serine residue. In this case, serine residues are not nucleophilic enough to be alkylated by a soft alkylating agent such as [urea-^{14}C]-4-*f*BCEU. SK-N-SH cells, which express significant amounts of neuronal-specific βIII-tubulin (33) as well as several other isoforms such as βI-, βII- and βIV-tubulin, were treated with [urea-^{14}C]-4-*f*BCEU. In contrast with other β-tubulin isoforms containing Cys239 (cf. **Fig. 5**), **Fig. 7A** shows that [urea-^{14}C]-4-*f*BCEU does not alter migration of the βIII-tubulin on SDS-PAGE and does not decrease cytosolic levels of βIII-tubulin. Moreover, the autoradiogram (Fig. 7B) shows that βIII-tubulin is not alkylated by [urea-^{14}C]-4-*f*BCEU since βIII-tubulin and the ^{14}C-labeled band are not co-localized on the gel. The ^{14}C-labeled band observed in **Fig. 7B** corresponds to other β-tubulin isoforms such as βI-, II- and βIV-tubulin. Taken together, these results suggest that the residu alkylated by CEU is most lik ly Cys239.

Findings

The present inv ntors have discovered the β-tubulin inhibiting properties of certain CEU's. Furthermore, it was discovered that these CEUs do not significantly alkylate nucleophiles such as DNA, glutathione and glutathione reductase, which are targeted by most commerically used alkylating agents such as nitrogen and phosphoramide mustards, nitrosoureas, methanesulfonate esters and aziridines (34, 35). The present invention shows that the cytotoxicity of these CEUs and their ability to selectively alkylate β-tubulin requires both weak alkylating properties and a hydrophobic character.

According to our structure-activity relationship studies, the aryl-3-(2-chloroethyl)urea moiety was found to be the pharmacophore responsible for the soft alkylating properties of CEU. The second portion of the molecule referred to as its "prosthetic moiety", is responsible for the hydrophobic properties of CEU and seems of utmost importance for the pharmacological activity of CEU on β-tubulin. Indeed, the pharmacophore *per se* is non-cytotoxic, whereas substitution of the aromatic ring at the 4-position by lower alkyl groups leads to cytotoxic CEU derivatives able to specifically alkylate β-tubulin. The kinetics of alkylation of β-tubulin suggest that nucleophilic addition of CEU requires a relatively long period of incubation. This type of kinetic behavior is probably related to several factors such as slow diffusion of the drugs into the cytosol and their weak alkylating properties, leading to slow nucleophilic addition. Nevertheless, the covalent binding of CEU to proteins seems specific and irreversible.

Furthermore, in the case of the *in vivo* alkylation of β-tubulin, the protein must be under its depolymerized form to react with CEU. The β-tubulin monomer, once alkylated, becomes incompetent for microtubule formation.

In a preferred embodiment, it was determined that the most likely reactive site of 4-*t*BCEU was ither Cys239 or Cys354, in the vicinity of the colchicine-binding site, since colchicine inhibits β-tubulin alkylation by CEU. Moreover, it was demonstrated that β-tubulin with a Cys → Ser substitution at position 239 is not

alkylat d by the drug, suggesting that Cys239 might be the residu alkylat d by a CEU such as 4-*t*BCEU. Previous evidence had established that Cys239, but not Cys354, is specifically alkylated by synthetic compounds such as 2,4-dichlorobenzyl thiocyanate (36) and 2-fluoro-1-methoxy-4-pentafluorophenyl-sulfonamidobenzene (37) inducing microtubule disassembly. These results suggest that Cys239 is more sensitive and more accessible to alkylation than Cys354. Thus, the integrity of Cys239 is most likely essential in the microtubule assembly process. However, the inventors cannot discard the possibility that 4-*t*BCEU alkylates β-tubulin at other residues on the protein, and that Cys239 is essential to maintain the proper conformation of β-tubulin reactive with CEU. Alkylation of β-tubulin by CEU induces the formation of a modified β-tubulin, which migrates ahead of native β-tubulin on SDS-PAGE. The electrophoretic behaviour of the modified β-tubulin obtained by alkylation of β-tubulin by CEU, is similar to the modified β-tubulin observed after the formation of a cross-link between Cys239 and Cys354 by EBI (32). It is important to mention that CEU are monoalkylating agents and are therefore unlikely to induce such cross-links in β-tubulin.

The probability that CEU could carbamoylate proteins through reaction of the carbonyl group of the urea moiety with lysine or cysteine residues in the vicinity of Cys239 or Cys354 is most unlikely. The chemical stability of aromatic ureas is very high and does not allow nucleophilic reactions, even with highly nucleophilic entities such as glutathione and glutathione reductase (data not shown). Moreover, there are no other nucleophilic entities present, either in the hydrophobic pocket or in the vicinity of the hydrophobic pocket that are available for such a reaction (25).

A plausible mechanistic explanation to the formation of modified β-tubulin by CEU is illustrated in **Fig. 8**. This putative mechanism of alkylation of β-tubulin by CEU is based on the analysis of the three-dimensional structure of β-tubulin, recently published by Nogales (25) and on the results of the present study. Briefly, the nucleophilic Cys239 is present in the drug-binding domain of colchicine, which is delimited by residues 206-381 that containing four mix d β-sheets and five surrounding α-helices. Two of the β-sheets, β8 and β9, are in close proximity,

leading to the formation of a hydrophobic pocket (~8Å across, between Cys239 and Cys354) (25). The existence of a hydrophobic pocket in tubulin was previously suggested as being in the vicinity of the colchicine binding site (38). This hydrophobic cavity could accommodate the aromatic ring, substituted by lower alkyl groups at the 4-position. It is speculated that the hydrophobic moiety of CEU readily forms hydrophobic bonds with several amino acid residues such as valine (316, 342, 349, 353), alanine (314, 315, 352) and leucine (240), present in the cavity. In addition, a strong hydrogen bond might be formed between the aromatic amino group of CEU and the glutamic acid residue at position 343. This hydrogen bond could be stabilized by electron resonance using the arginine residue at position 241.

After docking the CEU in the hydrophobic pocket, a hydrogen bond with the glutamic acid residue at position 343 would be formed; followed by an alkylation between the Cys239 residue and the 2-chloroethylamino moiety of CEU.

In summary, CEU are weak monoalkylating agents that are unreactive toward most cellular nucleophiles such as DNA, glutathion and glutathion reductase. On the other hand, CEUs were shown to alkylate specific cancer cell proteins bearing strong nucleophilic centers that present a spatial environment favoring close and prolonged contacts between the drug and the nucleophilic moiety.

These elements describe the concept of "soft alkylation", which introduces new perspectives about the rational design of drugs that might be able to inactivate specific cellular proteins with resulting cytotoxic effects directed at tumor cells.

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